(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 169 016 B1**

(12) # EUROPEAN PATENT SPECIFICATION

(45) Date of publication of patent specification: **04.10.95**

(51) Int. Cl.[6]: **C07K 14/00**, A61K 38/00, A61L 27/00

(21) Application number: **85304848.6**

(22) Date of filing: **08.07.85**

(54) **Polypeptide cartilage-inducing factors found in bone.**

(30) Priority: **16.07.84 US 630938**

(43) Date of publication of application:
22.01.86 Bulletin 86/04

(45) Publication of the grant of the patent:
04.10.95 Bulletin 95/40

(84) Designated Contracting States:
AT BE CH DE FR GB IT LI LU NL SE

(56) References cited:
EP-A- 0 182 483
WO-A-84/01106
US-A- 4 434 094
US-A- 4 440 750

PROCEEDNGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 80, November 1983, pages 6591-6595, National Academy of sciences of the USA, Washington, D.C. US; T.K.SAMPATH et al.

NATURE, vol. 316, 22nd August 1985, pages 701-705, Macmillan Journals Ltd,London, GB; R.DERYNCK et al.: " Human transforming growth factor-betacomplementary DNA sequence and expression in normal and transformed cells"

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EP 0 169 016 B1

Description

The present invention relates to protein chemistry. More particularly, it relates to two proteins that are found in bone, are co-factors for inducing cartilage formation, and are also active in the beta type transforming growth factor (TGF-β) assay. These polypeptides are sometimes referred to herein as cartilage-inducing factors (CIFs).

Human platelet/human placenta/bovine kidney-derived TGF-βs are described in International Patent Application WO-A-84/01106 and in EP-A-0128849.

U.S. 4,434,094 reports the partial purification of a bone generation-stimulating, bone-derived protein factor by extraction with chaotropic agents, fractionation on anion and cation exchange columns, and recovery of the activity from a fraction adsorbed to CMC at pH 4.8. This new protein fraction was termed "osteogenic factor" (OF) and was characterized as having a molecular weight below about 30,000 daltons and as tracking the purification process described. The proteins of the current invention were purified to homogeneity using a purification procedure that is similar in part to that disclosed in U.S. 4,434,094.

EP-A-105014 describes another TGF-β. The structure of one such, there designated TGF-β1 is elucidated and it is suggested that the TGF-β's could be used for wound healing. TGF-β1 is the polypeptide designated CIFA in the present application.

The invention provides a process for obtaining these polypeptides in substantially pure form from bone. Both CIFs are also active when combined with epidermal growth factor (EGF) in the TGF-β assay for in vitro induction of anchorage-independent growth of normal rat kidney (NRK) cells in soft agar. This assay is sometimes referred to herein as the TGF-β assay. In this regard the presence in bone of proteins having activity in the TGF-β assay has not been reported previously. One of the CIFs, designated CIF-A, has a partial (30 amino acids) N-terminal sequence that is identical to that reported in the literature for human placenta-derived TGF-β. The other CIF, designated CIF-B, has a partial N-terminal sequence that is different from the human placenta-derived TGF-β sequence and is claimed per se, and its structure elucidated, in the present invention.

Accordingly, one aspect of the invention is a polypeptide cartilage-inducing factor, which factor:

(a) is found in mammalian bone;

(b) is a co-factor for inducing cartilage formation;

(c) has activity in the TGF-β assay;

(d) is a dimer having an approximate molecular weight of 26,000 daltons as determined by SDS-PAGE;

(e) is isolatable by a process as claimed in claim 1 or claim 2; and

(f) does not have the N-terminal sequence:

Ala-Leu-Asp-Thr-Asn-Tyr-Cys-Phe-Ser(Ser)Thr-Glu-Lys-
Asn-Cys-Cys-Val-Arg-Gln-Leu-Tyr-Ile-Asp-Phe-Arg-Lys-
Asp-Leu-Gly-Trp-.

The process for isolating the two factors from bone is characterized by the following steps:

(a) treating demineralized bone (DMB) with a chaotropic (dissociative) extractant that solubilizes non-fibrous proteins;

(b) subjecting the extract from step (a) to gel filtration to recover a fraction containing proteins of molecular weight 10,000-40,000 daltons;

(c) adsorbing the fraction from step (b) onto a carboxymethyl cellulose cation exchanger at approximately pH 4.5-5.5 under denaturing conditions;

(d) eluting the adsorbed fraction from the cation exchanger with a sodium chloride gradient;

(e) subjecting the portion of the eluate from step (d) eluting at approximately 150 to 250 mM NaCl to reverse phase high performance liquid chromatography (RP-HPLC) or a nondenaturing gel electrophoresis; and

(f) recovering the factors from the RP-HPLC or gel electrophoresis.

An implant composition for inducing chondrogenesis/osteogenesis is characterized in that it contains an effective amount of CIFB, or both of the above-described CIFs, optionally together with a chondrogenic/osteogenic co-factor.

An implant composition for promoting connective tissue deposition is characterized in that it contains an effective amount of at least CIFB and is substantially free of any activating agent or chondrogenic co-factor.

Brief Description of the Drawings

In the drawings:

Figure 1 is a graph of the optical densities (absorbances) (280 nm) and in vitro chondrogenic activities of the gel filtration fractions of the example (section C), infra;

Figure 2 is a graph of the optical densities (280 nm) of eluate fractions from the preparative ion exchange chromatography of the example (section D), infra;

Figure 3 is a graph of the UV absorbance and electrophoretic profiles of peaks A (CIF-A) and B (CIF-B) of the preparative RP-HPLC of the example (section E), infra;

Figure 4 is a graph of the results of the enzyme-linked immunosorbent assays (ELISAs) for in vitro chondrogenic activity of the CIF-A and CIF-B obtained from the RP-HPLC of the example (section E), infra;

Figure 5 is a graph of the results of the ELISAs of the acid-urea gel electrophoresis fractions (section F) of the example, infra; and

Figure 6 is a graph of the results of the TGF-β assays described in section I of the example, infra.

Modes for Carrying Out the Invention

The polypeptides of the invention were isolated from bone. The polypeptides have been only partially sequenced at this time. In view of this and since the complete amino acid sequence of TGF-β has not been reported, the primary structure relationships between the CIFs of the invention and TGF-β are not known completely.

The polypeptides of the invention are co-factors for inducing cartilage formation. In view of their chondrogenic activity and the mode of endochondral bone formation, they are also expected to play a role in osteogenesis. The polypeptides are also active in the TGF-β assay and have been found to promote connective tissue deposition independently of association with TGF-β activating agents.

In view of the showings that bone inductive proteins from human, monkey, bovine and rat are nonspecies-specific in their abilities to produce endochondral bone in xenogeneic implants (Sampath, T. K., et al, Proc Natl Acad Sci (USA) (1983) 80:6591) and that human platelet/human placenta/bovine kidney-derived TGF-β is nonspecies-specific between rodents, cattle and humans, it is believed that the polypeptides of this invention have been highly conserved among mammalian species (i.e., polypeptides from different mammalian species have amino acid sequences that vary, if at all, in one or more amino acid residue additions, deletions, or substitutions that do not affect the nonspecies-specific activity of the molecule adversely). In this regard the term "substantially equivalent" as used to describe a polypeptide is intended to mean polypeptides, whether native or synthetic and regardless of species or derivation, that have the same amino acid sequence as a CIF, and polypeptides of substantially homologous but different amino acid sequence, which difference(s) does not affect nonspecies-specific activity adversely. Accordingly, the polypeptides may be derived from bone and perhaps other tissue of diverse animal origin or made by recombinant DNA technology. Porcine or bovine long bone are preferred native sources of the CIFs because of the ready availability of such bone and the high levels of the polypeptides in bone.

The procedure for isolating CIF from bone is as follows. The bone is first cleaned using mechanical or abrasive techniques, fragmented, and further washed with, for example, dilute aqueous acid preferably at low temperature, and then defatted by extraction with a lipophilic solvent such as ether or ethyl acetate. The bone is then demineralized by removal of the calcium phosphates in their various forms, usually by extraction with stronger acid. The resulting preparation, a demineralized bone, is the starting material for the preparation of the polypeptides of the invention.

The initial extraction is designed to remove the non-fibrous (e.g., non-collagenous) proteins from the demineralized bone. This can be done with the use of chaotropic agents such as guanidine hydrochloride (at least about 4 molar), urea (8 molar) plus salt, or sodium dodecylsulfate (at least about 1% by volume). The extraction is preferably carried out at reduced temperatures in the presence of a protease inhibitor to reduce the likelihood of digestion or denaturation of the extracted protein. Examples of protease inhibitors that may be included are phenylmethylsulfonylfluoride (PMSF) sodium azide, N-ethyl maleimide (NEM), benzamidine, and 6-aminohexanoic acid. The pH of the medium depends upon the extractant used. The process of extraction generally takes on the order of about 4 hr to one day.

After extraction, the extractant may be removed by suitable means such as dialysis against water, preceded by concentration by ultrafiltration if desired. Salts can also be removed by controlled electrophoresis or by molecular sieving. It is also preferred to maintain a low temperature during this process so as to minimize denaturation of the proteins. Alternatively, the extractant need not be removed, but rather the

solution need only be concentrated, for example, by ultrafiltration.

The extract, dissolved or redissolved in chaotropic agent, is subjected to gel filtration to obtain fractions of molecular weight below about 40,000 daltons, thus resulting in a major enhancement of purity. Gel sizing is done using standard techniques, preferably on a Sephacryl column at room (10-25 °C) temperature. The low molecular weight fraction is then subjected to ion exchange chromatography using carboxymethyl cellulose (CMC) at approximately pH 4.5-5.5, preferably about 4.8, in the presence of a nonionic chaotropic agent such as urea. Other cation exchangers may be used, including those derived from polyacrylamide and cross-linked dextran; however cellulosic cation exchangers are preferred. Of course, as in any ion exchange procedure, the solution must be freed of competing ions before application to the column, and is eluted in an increasing salt concentration gradient as is understood in the art. The fraction eluting from CMC at about 150 to 250 mM NaCl contains the CIFs.

The eluate fraction from the cation exchange chromatography is then subjected to RP-HPLC or a nondenaturing gel electrophoresis for final purification. Standard RP-HPLC techniques and gel electrophoresis techniques are used. Exemplified below is a commercially available RP-HPLC column using a commercially prescribed RP-HPLC protocol. This final purification yields the two polypeptides in substantially pure form. "Substantially pure" means that a polypeptide contains less than about 5% by weight contaminants.

Example

The following example is intended to illustrate the process for purification as applied to a particular sample. It is not intended to limit the invention.

A. Preparation of Demineralized Bone

Bovine metatarsal bone was obtained fresh from the slaughterhouse and transported on dry ice. The bones were cleaned of marrow and non-bone tissues, broken in fragments smaller than 1 cm diameter, and pulverized in a mill at 4 °C. The pulverized bone was washed twice with 9.4 liters of double distilled water per kg of bone for about 15 min each, and then washed overnight in 0.01 N HCl at 4 °C. Washed bone was defatted using 3 X 3 volumes ethanol, followed by 3 X 3 volumes diethylether, each washed for 20 min, and all at room temperature. The resulting defatted bone powder was then demineralized in 0.5 N HCl (25 l/kg defatted bone) at 4 °C. The acid was decanted and the resulting DMB washed until the wash pH was greater than 4, and the DMB dried on a suction filter.

B. Extraction of Noncollagenous Proteins

The DMB as prepared in paragraph A was extracted with 3.3 l of 4 M guanidine-HCl, 10 mM ethylenediaminetetraacetic acid (EDTA), pH 6.8, 1 mM PMSF, 10 mM NEM per kg for 16 hrs, the suspension suction filtered and the non-soluble material extracted again for 4 hrs. The soluble fractions were combined and concentrated at least 5-fold by ultrafiltration using an Amicon ultrafiltration (10K) unit, and the concentrate dialyzed against 6 changes of 35 volumes cold deionized water over a period of 4 days, and then lyophilized. All of the procedures of this paragraph were conducted at 4 °C except the lyophilization which was conducted under standard lyophilization conditions.

C. Gel Filtration

The extract from paragraph B, redissolved in 4 M guanidine-HCl, was fractionated on a Sephacryl S-200 column equilibrated in 4 M guanidine-HCl, 0.02% sodium azide, 10 mM EDTA, pH 6.8. Fractions were assayed by their absorbance at 280 nm and chondrogenic activity by ELISA (described below) and the fractions were combined as shown in Figure 1. Fraction F2 of Figure 1, constituting a low molecular weight (LMW, 10,000-40,000 daltons) protein fraction possessing the greatest activity was dialyzed against 6 changes of 180 volumes of deionized water and lyophilized. All operations except lyophilization and dialysis (4 °C) were conducted at room temperature.

D. Ion Exchange Chromatography

Fraction F2 from paragraph C was dissolved in 6 M urea, 10 mM NaCl, 1 mM NEM, 50 mM sodium acetate, pH 4.8 and centrifuged at 10,000 rpm for 5 min. The supernatant was fractionated on a CM52 (a

commercially available CMC) column equilibrated in the same buffer. Bound proteins were eluted from the column using a 10 mM to 400 mM NaCl gradient in the same buffer, and a total volume of 350 ml at a flow rate of 27 ml/hr. Three major fractions, designated CM-1, CM-2, and CM-3, were collected as shown in Figure 2. CM-2 and CM-3 were eluted at about 150 to 250 mM NaCl. Each fraction was dialyzed against 6 changes of 110 volumes of deionized water for 4 days and lyophilized. All of the foregoing operations were conducted at room temperature except dialysis (4 °C).

E. RP-HPLC

The lyophilized fractions CM-2 and CM-3 from ¶D were combined and dissolved in 0.1% trifluoroacetic acid (TFA) and aliquots of the solutions loaded onto a Vydac C18 RP-HPLC column (4.6 mm ID x 25 cm) and washed with 0.1% TFA for 5 min at 1 ml/min. The eluting solvent was a 0%-60% acetonitrile gradient in 0.1% TFA at a rate of 2%/min.

Two peaks were obtained from the RP-HPLC of combined CM-2 and CM-3--peak A at about 29.5 min and peak B at about 31.2 min. Figure 3 shows the absorbance and electrophoretic profiles (reduced and nonreduced) of peaks A and B. The proteins of these peaks were designated CIF-A and CIF-B, respectively.

The proteins were stored in 0.1% TFA/acetonitrile eluting solution at -20 °C until used.

F. Alternate Purification by Gel Electrophoresis

The combined lyophilized fractions CM-2 and CM-3 were fractionated by electrophoresis on an acetic acid-urea gel using the general procedure of Paynim, S. and Chalkley, R., Arch Bioch Biophys (1969) 130:337-346.

G. Assay for In Vitro Chondrogenic Activity

The presence of the desired protein in fractions during purification was confirmed using an in vitro assay for the production of cartilage-specific proteoglycans (PG), the identity of which was confirmed by ELISA. This assay is an agarose gel culture model using mesenchymal cells isolated from rat fetal muscle. It assesses the ability of the samples to induce the production of PG. The correlation between in vitro cartilage induction and in vivo bone formation has been shown by Seyedin, S., et al, J Cell Biol (1983) 97:1950-1953.

The cell culture was prepared by removing muscle tissue aseptically from the upper limbs of nineteen-day-old Sprague Dawley rat fetuses, mincing the tissue and culturing it in Eagle's Minimum Essential Medium (MEM) with 10% fetal bovine serum (FBS) and 50 units penicillin, 50 µg streptomycin per ml. Cellular outgrowth usually reached confluency within one week, whereupon cells were trypsinized, split 1:2 and used for experimentation within the first three passages.

The cells were placed in agarose gel cultures either with control medium or with samples to be tested. The procedure was basically that of Benya, et al, Cell (1982) 30:215. Briefly, the cell monolayers were harvested by trypsinization, counted on a hemocytometer, and resuspended at two times the final cell concentration in the medium with or without the protein fraction to be tested. The control medium was either Hams F-12, Dulbecco's Minimum Essential Medium (DMEM) or CMRL 1066 (Gibco) each containing 10% FBS and antibiotics. The test protein fractions in 0.01 N HCl were diluted directly to the desired concentration of test protein diluted with an equal volume with 1% low melting agarose (Bio-Rad, #162-0017) in F-12, and 0.2 ml of the dilution was plated on 17 mm wells coated with 0.15 ml of 1% high melting (Bio-Rad, #162-0100) agarose. The resulting cultures were incubated at 37 °C for 5 min, chilled at 4 °C for 10 min, and then overlayed with 1 ml of the corresponding medium (control or test protein). The cells were then cultured in a humidified atmosphere of 5% CO_2, 95% air and fed every 3-4 days thereafter by a complete change with control medium. After 7 days the cultures were frozen and stored at -80 °C before assay.

The cultures were assayed by thawing at 4 °C, homogenizing in 4 M guanidine-HCl with 50 nM Na acetate, 13 mM EDTA, 6 mM NEM, and 3 nM PMSF at pH 5.8, and extracting by shaking overnight at 4 °C. The supernatant fraction from centrifugation at 25,000 X g for 40 min at 4 °C was dialyzed overnight at 4 °C against 50 volumes 0.2 M NaCl, 50 mM Tris, pH 7.4. The supernatant was assayed for PG by ELISA as described by Renard, et al, Anal Biochem (1980) 104:205, and in U.S. 4,434,094.

Briefly, for the ELISA, antiserum to PG was raised in rabbits using standard techniques which showed no cross-reactivity with hyaluronic acid or PG extracted from rat bone. Purified PG (Seyedin, S., et al, supra) from Swarm rat chondrosarcoma tissue was used as standard antigen. The dialyzed samples were

diluted 1:1 (v/v) in phosphate-buffered saline (PBS) with 0.05% Tween 20, 1 mg/ml bovine serum albumin (BSA), pH 7.2 for assay. Horseradish peroxidase conjugated goat anti-rabbit IgG (Tago) was the second antibody with o-phenylenediamine as substrate.

The results of the ELISAs of CIF-A and CIF-B purified by RP-HPLC are shown in Figure 4. As indicated there, the sensitivity of the assay is within 1 to 5 ng/ml of culture media. The results of the ELISAs on the gel slices of section F are shown in Figure 5. These results are comparable to the results for CIF-A and CIF-B (corresponding to gel slices 7 and 6) from the RP-HPLC.

H. Characterization of Purified CIF-A and CIF-B

CIF-A was shown to be a 25,800 dalton protein which on reduction, yielded a 14,800 dalton polypeptide by measurements of the mobilities of the proteins in a 15% Laemmli polyacrylamide gel in SDS (Figure 3) as described by Laemmli, U. K., et al, Nature (1970) 227:680. It Is well understood that molecular weights so determined are approximate and their values are dependent on the method used. The conformation of the protein affects its mobility in this system, and, therefore, the molecular weights obtained will be similar, but not necessarily identical when determined by other procedures. The presence of a single band in the profile of the reduced protein indicates the protein is probably a dimer composed of two polypeptide chains having substantially equivalent amino acid sequences (i.e., it is a homodimer). The discrepancy between the measured weights of dimer and the individual chains is an artifact of the procedure.

CIF-A maintained its activity in the ELISA assay of paragraph G above even after heating for 3 min at 100 °C in PBS, after treatment with collagenase for 2 hrs at 37 °C in 0.1 M Tris, pH 7.4, 5 mM $CaCl_2$, 0.02 mM PMSF with a ratio of collagenase to protein of 400 units/mg protein, and after treatment with trypsin for 2 hrs at 37 °C in 50 mM Tris, pH 7.4, 10 mM $CaCl_2$ with a ratio of trypsin to protein of 100 units/mg of protein. However, the protein lost activity after treatment for 1 hr at room temperature in PBS containing 5 mM dithiothreitol (DTT), which would effect reduction of disulfide linkages. Similarly, SDS treatment or fractionation on SDS-PAGE resulted in inactivation of the protein, presumably due to denaturation or complexing by the SDS. The partial amino acid composition of CIF-A is shown in Table 1.

Table 1

Amino Acid	Mols/100 Mols Recovered
Asp	9.2
Glu	9.2
Ser	7.0
His	2.7
Gly	16.5
Thr	2.7
Arg	5.9
Ala	6.6
Tyr	3.2
Met	0.0
Val	7.5
Phe	3.0
Ile	3.9
Leu	8.6
Lys	13.9
Pro	ND
Cys	ND
Trp	ND

Amino acid sequence analysis of CIF-A showed that it has the following single N-terminal sequence:

```
   1                5                  10
Ala-Leu-Asp-Thr-Asn-Tyr-Cys-Phe-Ser(Ser)Thr-Glu-Lys-Asn-

  15                20                  25
-Cys-Cys-Val-Arg-Gln-Leu-Tyr-Ile-Asp-Phe-Arg-Lys-Asp-Leu-

      30
-Gly-Trp-.
```

( ) = suspected

This N-terminal sequence is identical to that reported for human placenta-derived TGF-β.

CIF-B had a slightly different molecular weight (26,000) as measured by the same procedure. This difference may be caused by the procedure. Accordingly, both proteins are considered to have a molecular weight of approximately 26,000 daltons as measured by SDS-PAGE. On reduction the protein of peak B showed a single band at approximately 14,200 daltons indicating that it, too, is probably a homodimer. It has the amino acid composition set forth in Table 2.

Table 2

Amino Acid	Mols/100 Mols Recovered
Asp	12.0
Glu	8.5
Ser	10.6
His	0.9
Gly	22.0
Thr	0.0
Arg	4.3
Ala	6.7
Tyr	1.9
Met	0.0
Val	2.4
Phe	3.0
Ile	2.2
Leu	8.2
Lys	17.3
Pro	ND
Cys	ND
Trp	ND

Amino acid sequence analysis indicated CIF-B has a single N-terminal sequence as follows:

```
     1                   5                  10
Ala-Leu-Asp-Ala-Ala-Tyr-Cys-Phe-Arg-Asn-Val-Glu-Asp-Asn-

     15                  20                  25
-Cys-Cys-Leu-Arg-Pro-Leu-Tyr-Ile-Asp-Phe-Lys-Arg-Asp-Leu-

     30
-Gly-Trp-.
```

Its other properties, as qualitatively assessed, were similar to those set forth above for CIF-A.

I. Assay for TGF-β Activity

CIF-A and CIF-B were tested in the TGF-β bioassay. The assay was performed as described in Methods for Preparation of Media, Supplements, and Substrata for Serum-Free Animal Cell Culture (1984) pp 181-194, Alan R. Liss, Inc. The results of the assay are shown in Figure 6. As depicted, both proteins exhibit a clear dose-response in the assay and require the presence of an activating agent (EGF) to be active. The levels of activity are comparable to the reported levels of activity of human platelet/human placenta/bovine kidney-derived TGF-β.

The ability of the CIFs to withstand treatment with trypsin without losing activity may make it possible to isolate them from demineralized bone powder by means of enzymatic digestion. In such a process the demineralized bone powder is digested with an aqueous solution of trypsin and/or other proteases that do not degrade the proteins of interest under conditions at which such enzymes are active. This treatment digests the majority of other protein components in the powder. The proteins of interest may be purified from the resulting digest using one or more of the fractionation techniques described above (gel filtration, ion exchange chromatography, RP-HPLC or nondenaturing gel electrophoresis). Depending upon the extent to which the CIFs are released from the bone matrix and not complexed with other materials, use of solubilizing agents may be avoided. In this regard the pure proteins are substantially soluble in water.

The CIFs are useful for inducing cartilage/bone growth for repairing, replacing or augmenting cartilage/bone tissue in animals, including humans. Chondrogenically/osteogenically effective amounts of the proteins may be combined with chondrogenic/osteogenic co-factors found in bone and formulated with pharmacologically and physiologically acceptable fluid or solid carriers such as purified collagen for implantation. The weight ratio of active protein to carrier will typically be in the range of 1:50 to 1:1000. The implants may be placed at a predetermined site in the patient by conventional surgical techniques, including injection as an active ingredient. Collagenous implants containing only CIFB as an active ingredient (i.e., free of any activating agent or co-factor) at CIF to carrier weight ratios above about 1:6000 promoted collagenous connective tissue deposition.

The CIFs may also be used in the same manner as human platelet/human placenta/bovine kidney-derived TGF-β to promote (provoke and sustain) non-species specific cellular proliferation. In such application one or both of the CIFs is combined in approximately stoichiometric proportions with a TGF-β activating agent such as an EGF or a TGF-α. Clinical applications of the cell proliferation activity of these compositions include topical administration for burn or wound healing, implantation for tissue augmentation, and systemic administration for internal wound healing. In such uses the CIF and activating agent will be formulated in amounts sufficient to induce cell proliferation with pharmaceutically acceptable carriers that are adapted for the particular mode of administration. Topical dosage forms will typically be formulated as sprays, gels, ointments, or salves. Implants will be formulated as injectables. Systemic dosage forms may be formulated for enteral administration (e.g., liquids, pills, tablets) or for parenteral injection. The dosages used in such applications cannot be specified because of the nature of cell proliferation and the variability in wounds and other traumata.

Claims

Claims for the following Contracting States : GB, DE, FR, IT, NL, SE, CH, BE, LU

1. A process for isolating a polypeptide cartilage-inducing factor from bone, which factor:
   (a) is found in mammalian bone;
   (b) is a co-factor for inducing cartilage formation;
   (c) has activity in the TGF-β assay; and
   (d) is a dimer having an approximate molecular weight of 26,000 daltons as determined by SDS-PAGE;
   the process comprising:
   (i) treating demineralized bone with a chaotropic extractant that solubilizes nonfibrous proteins;
   (ii) subjecting the extract from step (i) to gel filtration to recover a fraction containing proteins of molecular weight 10,000-40,000 daltons;
   (iii) adsorbing the fraction from step (ii) onto a carboxymethyl cellulose cation exchanger at approximately pH 4.5-5.5 under denaturing conditions;
   (iv) eluting the adsorbed fraction from the cation exchanger with a sodium chloride gradient;
   (v) subjecting the portion of the eluate of (iv) that elutes at approximately 150 to 250 mM sodium chloride to RP-HPLC or a nondenaturing gel electrophoresis; and
   (vi) recovering said factor from the RP-HPLC or nondenaturing gel electrophoresis.

2. A process according to claim 1 wherein the bone is bovine bone.

3. A process according to claim 1 or 2 wherein each chain of the dimer has an N-terminal sequence substantially as follows:

```
Ala-Leu-Asp-Thr-Asn-Tyr-Cys-Phe-Ser(Ser)Thr-Glu-Lys-
Asn-Cys-Cys-Val-Arg-Gln-Leu-Tyr-Ile-Asp-Phe-Arg-Lys-
Asp-Leu-Gly-Trp-.
```

4. A polypeptide cartilage-inducing factor, which factor:
   (a) is found in mammalian bone;
   (b) is a co-factor for inducing cartilage formation;
   (c) has activity in the TGF-β assay;
   (d) is a dimer having an approximate molecular weight of 26,000 daltons as determined by SDS-PAGE;
   (e) is isolatable by a process according to claim 1 or claim 2; and
   (f) does not have the N-terminal sequence

```
Ala-Leu-Asp-Thr-Asn-Tyr-Cys-Phe-Ser(Ser)Thr-Glu-Lys-
Asn-Cys-Cys-Val-Arg-Gln-Leu-Tyr-Ile-Asp-Phe-Arg-Lys-
Asp-Leu-Gly-Trp-.
```

5. A factor according to claim 4 wherein the bone of (a) is bovine bone.

6. An implant composition for inducing chondrogenesis/osteogenesis which contains at least one factor according to claim 4 or 5.

7. An implant composition for promoting connective tissue deposition which contains at least one factor according to claims 4 or 5 and which is substantially free of any activating agent or co-factor.

8. A composition for inducing chondrogenesis or osteogenesis which contains
(a) at least one factor according to claim 4 or 5; and
(b) a chondrogenic or osteogenic co-factor.

9. Use of a factor of claim 4 or claim 5 in the manufacture of an implant composition for inducing chondrogenesis/osteogenesis or for promoting connective tissue deposition.

Claims for the following Contracting State : AT

1. A process for isolating a polypeptide cartilage-inducing factor from bone, which factor:
(a) is found in mammalian bone;
(b) is a co-factor for inducing cartilage formation;
(c) has activity in the TGF-β assay; and
(d) is a dimer having an approximate molecular weight of 26,000 daltons as determined by SDS-PAGE;
the process comprising:
(i) treating demineralized bone with a chaotropic extractant that solubilizes nonfibrous proteins;
(ii) subjecting the extract from step (i) to gel filtration to recover a fraction containing proteins of molecular weight 10,000-40,000 daltons;
(iii) adsorbing the fraction from step (ii) onto a carboxymethyl cellulose cation exchanger at approximately pH 4.5-5.5 under denaturing conditions;
(iv) eluting the adsorbed fraction from the cation exchanger with a sodium chloride gradient;
(v) subjecting the portion of the eluate of (iv) that elutes at approximately 150 to 250 mM sodium chloride to RP-HPLC or a nondenaturing gel electrophoresis; and
(vi) recovering said factor from the RP-HPLC or nondenaturing gel electrophoresis.

2. A process according to claim 1 wherein the bone is bovine bone.

3. A process according to claim 1 or 2 wherein each chain of the dimer has an N-terminal sequence substantially as follows:

Ala-Leu-Asp-Thr-Asn-Tyr-Cys-Phe-Ser(Ser)Thr-Glu-Lys-
Asn-Cys-Cys-Val-Arg-Gln-Leu-Tyr-Ile-Asp-Phe-Arg-Lys-
Asp-Leu-Gly-Trp-.

4. A process according to claim 1 or 2 wherein each chain of the dimer has not got the N-terminal sequence substantially as follows:

Ala-Leu-Asp-Thr-Asn-Tyr-Cys-Phe-Ser(Ser)Thr-Glu-Lys-
Asn-Cys-Cys-Val-Arg-Gln-Leu-Tyr-Ile-Asp-Phe-Arg-Lys-
Asp-Leu-Gly-Trp-.

5. A process for producing an implant composition for inducing chondrogenesis/osteogenesis, which composition contains at least one factor produced according to claim 4.

6. A process for producing an implant composition for promoting connective tissue deposition, which comprises forming the composition to contain at least one factor produced according to claim 4 but to be substantially free of any activating agent or co-factor.

7. A process for producing a composition for inducing chondrogenesis or osteogenesis comprising forming the composition to contain:
   (a) at least one factor produced according to claim 4; and
   (b) a chondrogenic or osteogenic co-factor.

8. Use of a factor produced according to claim 4 in the manufacture of an implant composition for inducing chondrogenesis/osteogenesis or for promoting connective tissue deposition.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : GB, DE, FR, IT, NL, SE, CH, BE, LU

1. Verfahren zum Isolieren eines knorpel-induzierenden Polypeptid-Faktors aus Knochen, wobei der Faktor
   (a) in Säugetierknochen anzutreffen ist;
   (b) ein Co-Faktor zum Induzieren von Knorpelbildung ist;
   (c) im TGF-β-Assay Aktivität zeigt; und
   (d) ein Dimer mit einem ungefähren Molekulargewicht von 26.000 Dalton ist, wie durch SDS-PAGE bestimmt;
   wobei das Verfahren umfaßt:
   (i) das Behandeln von entmineralisierten Knochen mit einem chaotropen Extraktionsmittel, das nicht-fibröse Proteine solubilisiert;
   (ii) das Unterwerfen des Extrakts aus Schritt (i) einer Gelfiltration, um eine Fraktion zu gewinnen, die Proteine mit einem Molekulargewicht von 10.000 - 40.000 Dalton enthält;
   (iii) das Adsorbieren der Fraktion aus Schritt (ii) an einen Carboxymethylzellulose-Kationenaustauscher bei pH = 4,5 - 5,5 unter denaturierenden Bedingungen;
   (iv) das Eluieren der adsorbierten Fraktion vom Kationenaustauscher mittels eines NaCl-Gradienten;
   (v) das Unterziehen jenes Anteils des Eluats aus (iv), der bei etwa 150 - 250 mMol NaCl eluiert, einer RP-HPLC oder einer nicht-denaturierenden Gelelktrophorese; und
   (vi) das Gewinnen des Faktors aus der RP-HPLC oder der nicht-denaturierenden Gelelktrophorese.

2. Verfahren nach Anspruch 1, worin die Knochen Rinderknochen sind.

3. Verfahren nach Anspruch 1 oder 2, worin jede der Dimer-Ketten eine N-terminale Sequenz im wesentlichen wie folgt aufweist:

Ala-Leu-Asp-Thr-Asn-Tyr-Cys-Phe-Ser(Ser)Thr-Glu-Lys-

Asn-Cys-Cys-Val-Arg-Gln-Leu-Tyr-Ile-Asp-Phe-Arg-Lys-

Asp-Leu-Gly-Trp-.

4. Knorpel-induzierender Polypeptid-Faktor, der
   (a) in Säugetierknochen anzutreffen ist;
   (b) ein Co-Faktor zum Induzieren von Knorpelbildung ist;
   (c) im TGF-β-Assay Aktivität zeigt;
   (d) ein Dimer mit einem ungefähren Molekulargewicht von 26.000 Dalton ist, wie durch SDS-PAGE bestimmt;
   (e) nach einem Verfahren nach Anspruch 1 oder 2 isolierbar ist; und

(f) nicht die N-terminale Sequenz

Ala-Leu-Asp-Thr-Asn-Tyr-Cys-Phe-Ser(Ser)Thr-Glu-Lys-
Asn-Cys-Cys-Val-Arg-Gln-Leu-Tyr-Ile-Asp-Phe-Arg-Lys-
Asp-Leu-Gly-Trp-

aufweist.

5. Faktor nach Anspruch 4, worin die Knochen unter (a) Rinderknochen sind.

6. Implantat-Zusammensetzung zur Induktion von Chondrogenese/Osteogenese, die zumindest einen Faktor nach Anspruch 4 oder 5 enthält.

7. Implantat-Zusammensetzung zur Förderung von Bindegewebe-Ablagerung, die zumindest einen Faktor nach Anspruch 4 oder 5 enthält und im wesentlichen frei von jeglichem Aktivator oder Co-Faktor ist.

8. Zusammensetzung zur Induktion von Chondrogenese oder Osteogenese, die enthält:
(a) zumindest einen Faktor nach Anspruch 4 oder 5; und
(b) einen Chondrogenese- oder Osteogenese-Co-Faktor.

9. Verwendung eines Faktors nach Anspruch 4 oder 5 zur Herstellung einer Implantat-Zusammensetzung zur Induktion von Chondrogenese/Osteogenese oder zur Förderung von Bindegewebe-Ablagerung.

Patentansprüche für folgenden Vertragsstaat : AT

1. Verfahren zum Isolieren eines knorpel-induzierenden Polypeptid-Faktors aus Knochen, wobei der Faktor
(a) in Säugetierknochen anzutreffen ist;
(b) ein Co-Faktor zum Induzieren von Knorpelbildung ist;
(c) im TGF-β-Assay Aktivität zeigt; und
(d) ein Dimer mit einem ungefähren Molekulargewicht von 26.000 Dalton ist, wie durch SDS-PAGE bestimmt;
wobei das Verfahren umfaßt:
(i) das Behandeln von entmineralisierten Knochen mit einem chaotropen Extraktionsmittel, das nicht-fibröse Proteine solubilisiert;
(ii) das Unterwerfen des Extrakts aus Schritt (i) einer Gelfiltration, um eine Fraktion zu gewinnen, die Proteine mit einem Molekulargewicht von 10.000 - 40.000 Dalton enthält;
(iii) das Adsorbieren der Fraktion aus Schritt (ii) an einen Carboxymethylzellulose-Kationenaustauscher bei pH = 4,5 - 5,5 unter denaturierenden Bedingungen;
(iv) das Eluieren der adsorbierten Fraktion vom Kationenaustauscher mittels eines NaCl-Gradienten;
(v) das Unterziehen jenes Anteils des Eluats aus (iv), der bei etwa 150 - 250 mMol NaCl eluiert, einer RP-HPLC oder einer nicht-denaturierenden Gelelktrophorese; und
(vi) das Gewinnen des Faktors aus der RP-HPLC oder der nicht-denaturierenden Gelelktrophorese.

2. Verfahren nach Anspruch 1, worin die Knochen Rinderknochen sind.

3. Verfahren nach Anspruch 1 oder 2, worin jede der Dimer-Ketten eine N-terminale Sequenz im wesentlichen wie folgt aufweist:

Ala-Leu-Asp-Thr-Asn-Tyr-Cys-Phe-Ser(Ser)Thr-Glu-Lys-
Asn-Cys-Cys-Val-Arg-Gln-Leu-Tyr-Ile-Asp-Phe-Arg-Lys-
Asp-Leu-Gly-Trp-.

4. Verfahren nach Anspruch 1 oder 2, worin keine der Dimer-Ketten eine N-terminale Sequenz im wesentlichen wie folgt aufweist:

Ala-Leu-Asp-Thr-Asn-Tyr-Cys-Phe-Ser(Ser)Thr-Glu-Lys-
Asn-Cys-Cys-Val-Arg-Gln-Leu-Tyr-Ile-Asp-Phe-Arg-Lys-
Asp-Leu-Gly-Trp-.

5. Verfahren zur Herstellung einer Implantat-Zusammensetzung zur Induktion von Chondrogenese/Osteogenese, die zumindest einen nach Anspruch 4 hergestellten Faktor enthält.

6. Verfahren zur Herstellung einer Implantat-Zusammensetzung zur Förderung von Bindegewebe-Ablagerung, das die Bildung der Zusammensetzung umfaßt, die zumindest einen nach Anspruch 4 hergestellten Faktor enthält und im wesentlichen frei von jeglichem Aktivator oder Co-Faktor ist.

7. Verfahren zur Herstellung einer Zusammensetzung zur Induktion von Chondrogenese oder Osteogenese, das die Bildung der Zusammensetzung umfaßt, die enthält:
   (a) zumindest einen nach Anspruch 4 hergestellten Faktor; und
   (b) einen Chondrogenese- oder Osteogenese-Co-Faktor.

8. Verwendung eines nach Anspruch 4 hergestellten Faktors zur Herstellung einer Implantat-Zusammensetzung zur Induktion von Chondrogenese/Osteogenese oder zur Förderung von Bindegewebe-Ablagerung.

Revendications
Revendications pour les Etats contractants suivants : GB, DE, FR, IT, NL, SE, CH, BE, LU

1. Procédé d'isolement d'un facteur polypeptidique, induisant le cartilage, de l'os, lequel facteur :
   (a) est trouvé dans l'os mammalien ;
   (b) est un co-facteur pour induire la formation de cartilage ;
   c) a une activité dans le dosage de TGF-β ; et
   (d) est un dimère ayant un poids moléculaire approximatif de 26 000 daltons comme déterminé par SDS-PAGE ;
   le procédé consistant à :
   (i) traiter l'os déminéralisé avec un agent d'extraction chaotrope qui solubilise les protéines non-fibreuses ;
   (ii) soumettre l'extrait de l'étape (i) à une filtration sur gel pour récupérer une fraction contenant des protéines de poids moléculaire de 10 000 à 40 000 daltons ;
   (iii) adsorber la fraction de l'étape (ii) sur un échangeur de cations de carboxyméthylcellulose à un pH d'approximativement 4,5 à 5,5 en conditions dénaturantes ;
   (iv) éluer la fraction adsorbée de l'échangeur de cations avec un gradient de chlorure de sodium ;
   (v) soumettre la portion de l'éluat de (iv) qui s'élue à approximativement 150 à 250 mM de chlorure de sodium à une RP-HPLC ou à une électrophorèse sur gel non-dénaturante ; et
   (vi) récupérer ledit facteur de la RP-HPLC ou de l'électrophorèse sur gel non-dénaturante.

2. Procédé selon la revendication 1 dans lequel l'os est de l'os bovin.

3. Procédé selon la revendication 1 ou 2 dans lequel chaque chaîne du dimère a une séquence N-terminale substantiellement comme suit :

Ala-Leu-Asp-Thr-Asn-Tyr-Cys-Phe-Ser(Ser)Thr-Glu-Lys-
Asn-Cys-Cys-Val-Arg-Gln-Leu-Tyr-Ile-Asp-Phe-Arg-Lys-
Asp-Leu-Gly-Trp-.

4. Facteur polypeptidique induisant du cartilage, lequel facteur :
(a) est trouvé dans l'os mammalien ;
(b) est un co-facteur pour induire la formation de cartilage ;
(c) a une activité dans le dosage de TGF-β ;
(d) est un dimère ayant un poids moléculaire d'approximativement 26 000 daltons comme déterminé par SDS-PAGE ;
(e) est isolable par un procédé selon la revendication 1 ou la revendication 2 ; et
(f) n'a pas la séquence N-terminale

Ala-Leu-Asp-Thr-Asn-Tyr-Cys-Phe-Ser(Ser)Thr-Glu-Lys-
Asn-Cys-Cys-Val-Arg-Gln-Leu-Tyr-Ile-Asp-Phe-Arg-Lys-
Asp-Leu-Gly-Trp-.

5. Facteur selon la revendication 4 dans lequel l'os de (a) est un os bovin.

6. Composition d'implant pour induire une chondrogénèse/ostéogénèse qui contient au moins un facteur selon la revendication 4 ou 5.

7. Composition d'implant pour provoquer un dépôt de tissu conjonctif qui contient au moins un facteur selon les revendications 4 ou 5 et qui est substantiellement exempte de tout agent ou co-facteur activateur.

8. Composition pour induire la chondrogénèse ou l'ostéogénèse qui contient
(a) au moins un facteur selon la revendication 4 ou 5 ; et
(b) un co-facteur chondrogène ou ostéogène.

9. Utilisation d'un facteur selon la revendication 4 ou la revendication 5 dans la fabrication d'une composition d'implant pour induire la chondrogénèse/ostéogénèse ou pour provoquer un dépôt de tissu conjonctif.

Revendications pour l'Etat contractant suivant : AT

1. Procédé d'isolement d'un facteur polypeptidique induisant le cartilage de l'os, lequel facteur :
(a) est trouvé dans l'os mammalien ;
(b) est un co-facteur pour induire la formation de cartilage ;
c) a une activité dans le dosage de TGF-β ; et
(d) est un dimère ayant un poids moléculaire approximatif de 26 000 daltons comme déterminé par SDS-PAGE ;
le procédé consistant à :
(i) traiter l'os déminéralisé avec un agent d'extraction chaotrope qui solubilise les protéines non-fibreuses ;
(ii) soumettre l'extrait de l'étape (i) à une filtration sur gel pour récupérer une fraction contenant des protéines de poids moléculaire de 10 000 à 40 000 daltons ;

(iii) adsorber la fraction de l'étape (ii) sur un échangeur de cations de carboxyméthylcellulose à un pH d'approximativement 4,5 à 5,5 en conditions dénaturantes ;
(iv) éluer la fraction adsorbée de l'échangeur de cations avec un gradient de chlorure de sodium ;
(v) soumettre la portion de l'éluat de (iv) qui s'élue à approximativement 150 à 250 mM de chlorure de sodium à une RP-HPLC ou à une électrophorèse sur gel non-dénaturante ; et
(vi) récupérer ledit facteur de la RP-HPLC ou de l'électrophorèse sur gel non-dénaturante.

2. Procédé selon la revendication 1 dans lequel l'os est de l'os bovin.

3. Procédé selon la revendication 1 ou 2 dans lequel chaque chaîne du dimère a une séquence N-terminale substantiellement comme suit :

Ala-Leu-Asp-Thr-Asn-Tyr-Cys-Phe-Ser(Ser)Thr-Glu-Lys-
Asn-Cys-Cys-Val-Arg-Gln-Leu-Tyr-Ile-Asp-Phe-Arg-Lys-
Asp-Leu-Gly-Trp-.

4. Procédé selon la revendication 1 ou 2 dans lequel chaque chaîne du dimère n'a pas la séquence N-terminale substantiellement comme suit :

Ala-Leu-Asp-Thr-Asn-Tyr-Cys-Phe-Ser(Ser)Thr-Glu-Lys-
Asn-Cys-Cys-Val-Arg-Gln-Leu-Tyr-Ile-Asp-Phe-Arg-Lys-
Asp-Leu-Gly-Trp-.

5. Procédé de production d'une composition d'implant pour induire une chondrogénèse/ostéogénèse, laquelle composition contient au moins un facteur produit selon la revendication 4.

6. Procédé de production d'une composition d'implant pour provoquer un dépôt de tissu conjonctif, qui comprend la formation de la composition pour contenir au moins un facteur produit selon la revendication 4 mais pour être substantiellement libre de tout agent ou co-facteur activateur.

7. Procédé de production d'une composition pour induire une chondrogénèse ou une ostéogénèse comprenant la formation de la composition pour contenir :
(a) au moins un facteur produit selon la revendication 4 ; et
(b) un co-facteur chondrogène ou ostéogène.

8. Utilisation d'un facteur produit selon la revendication 4 dans la fabrication d'une composition d'implant pour induire une chondrogénèse/ostéogénèse ou pour provoquer un dépôt de tissu conjonctif.

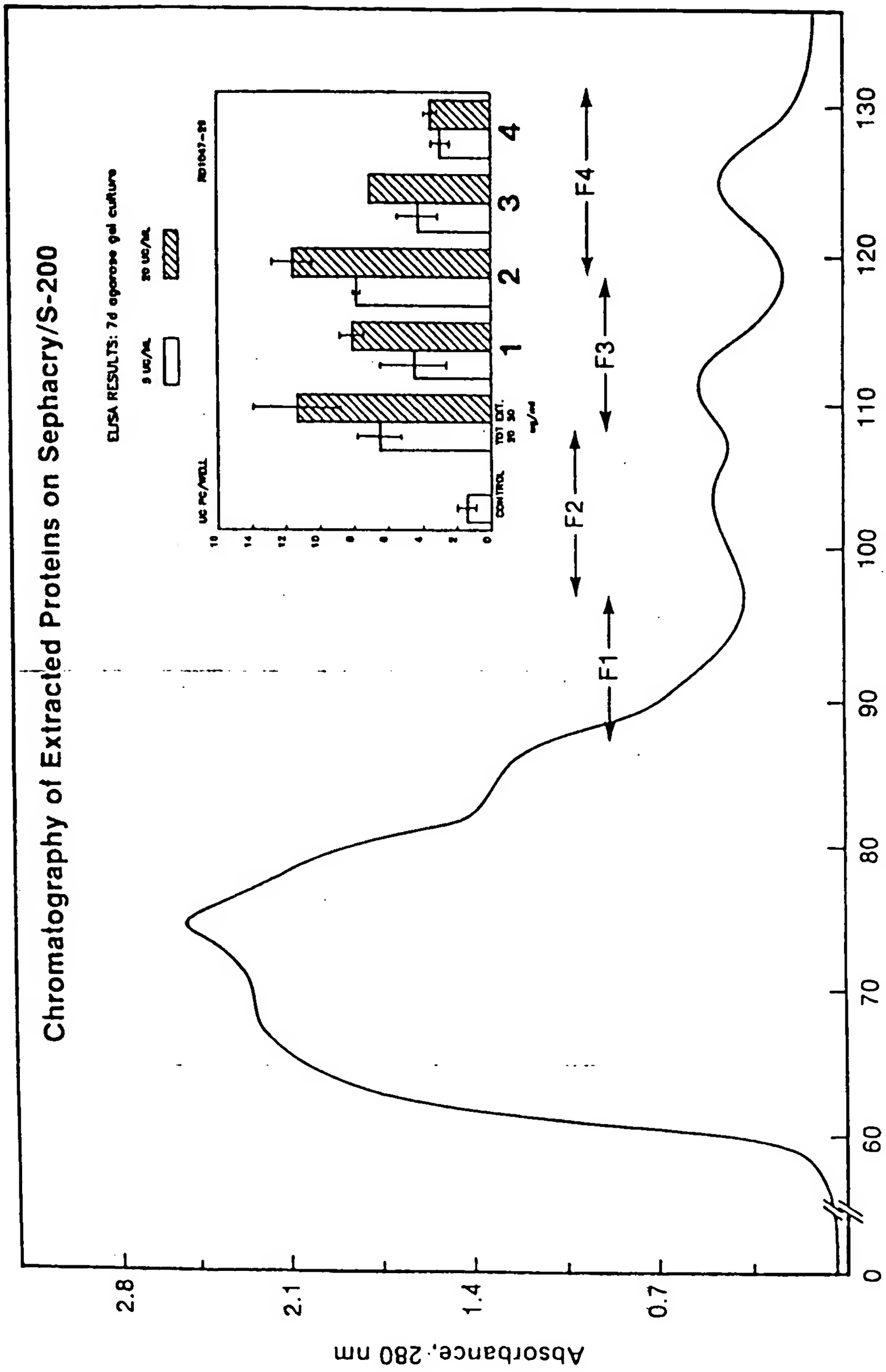
Chromatography of Extracted Proteins on Sephacry/S-200
ELISA RESULTS: 7d agarose gel culture
5 UC/ML
20 UC/ML
RD1047-88
UC PC/WELL
CONTROL
F1
F2
F3
F4
Absorbance, 280 nm
2.8
2.1
1.4
0.7
0
60
70
80
90
100
110
120
130
Fraction Number

FIG. 1

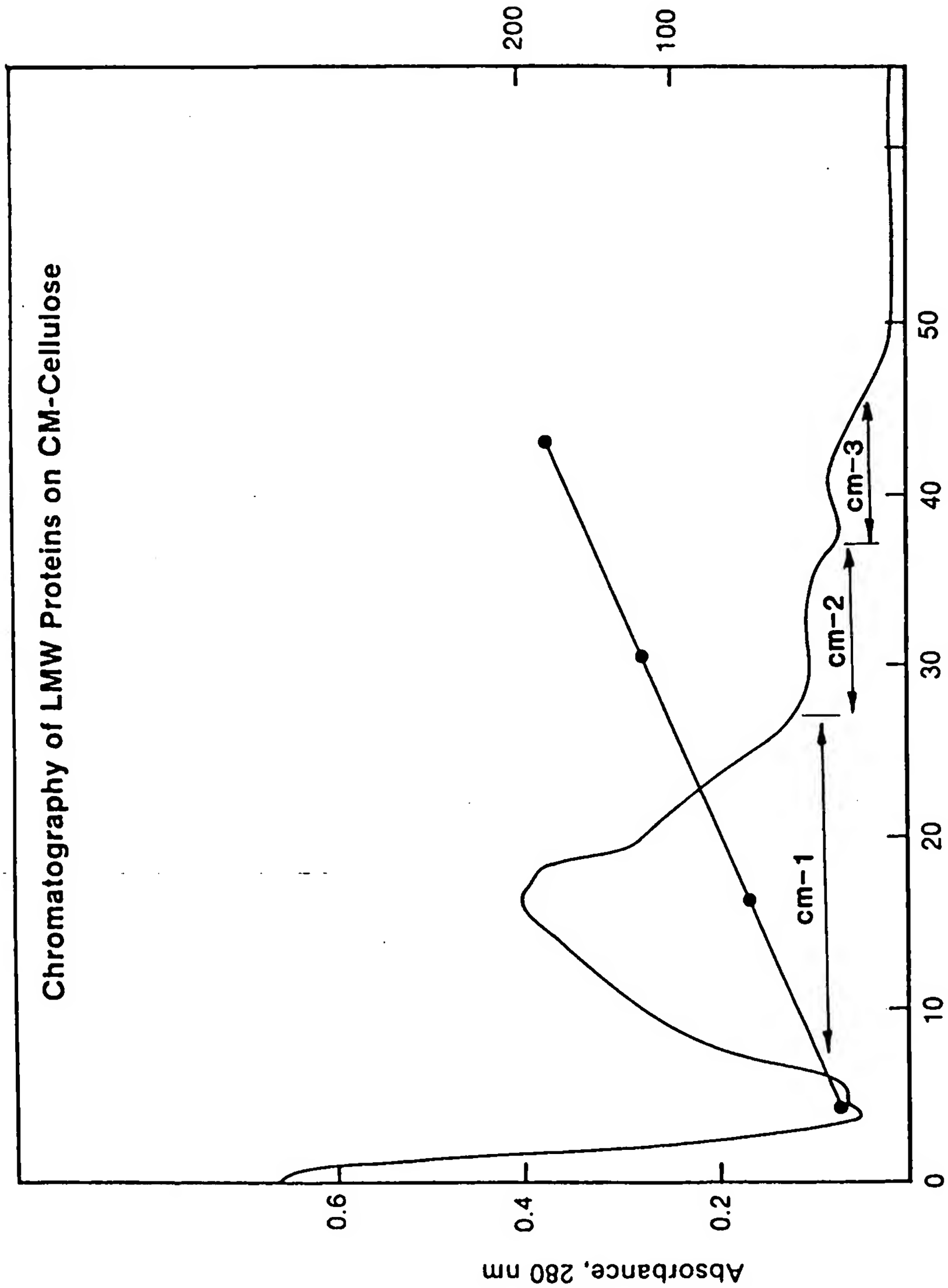
Chromatography of LMW Proteins on CM-Cellulose
Absorbance, 280 nm
Fraction Number
cm-1
cm-2
cm-3
0
10
20
30
40
50
100
200
0.2
0.4
0.6

FIG. 2

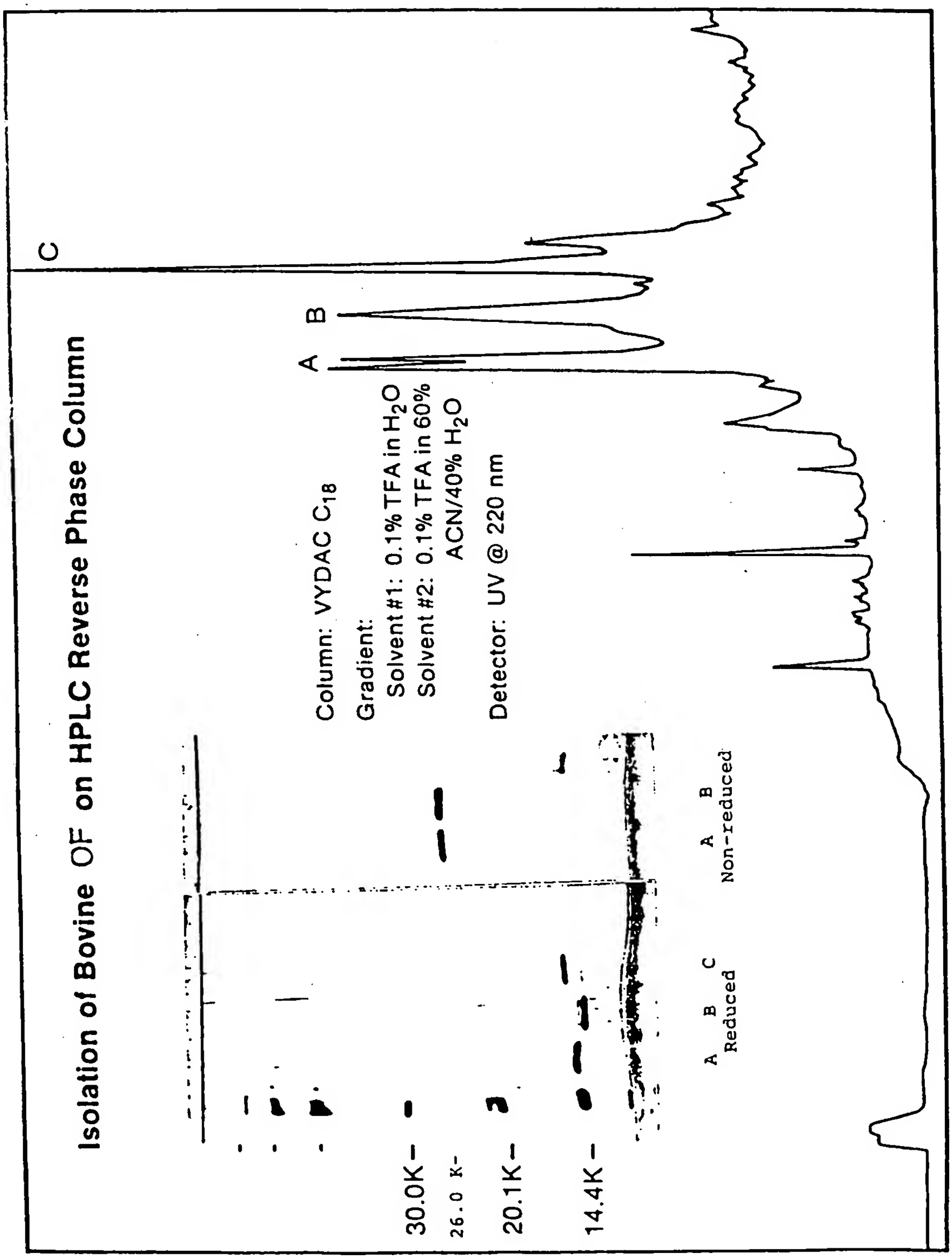
Isolation of Bovine C OF on HPLC Reverse Phase Column
C
A B
Column: VYDAC C_{18}
Gradient:
Solvent #1: 0.1% TFA in H_2O
Solvent #2: 0.1% TFA in 60% ACN/40% H_2O
Detector: UV @ 220 nm
30.0K
26.0 K
20.1K
14.4K
A B
Non-reduced
A B C
Reduced

FIG. 3

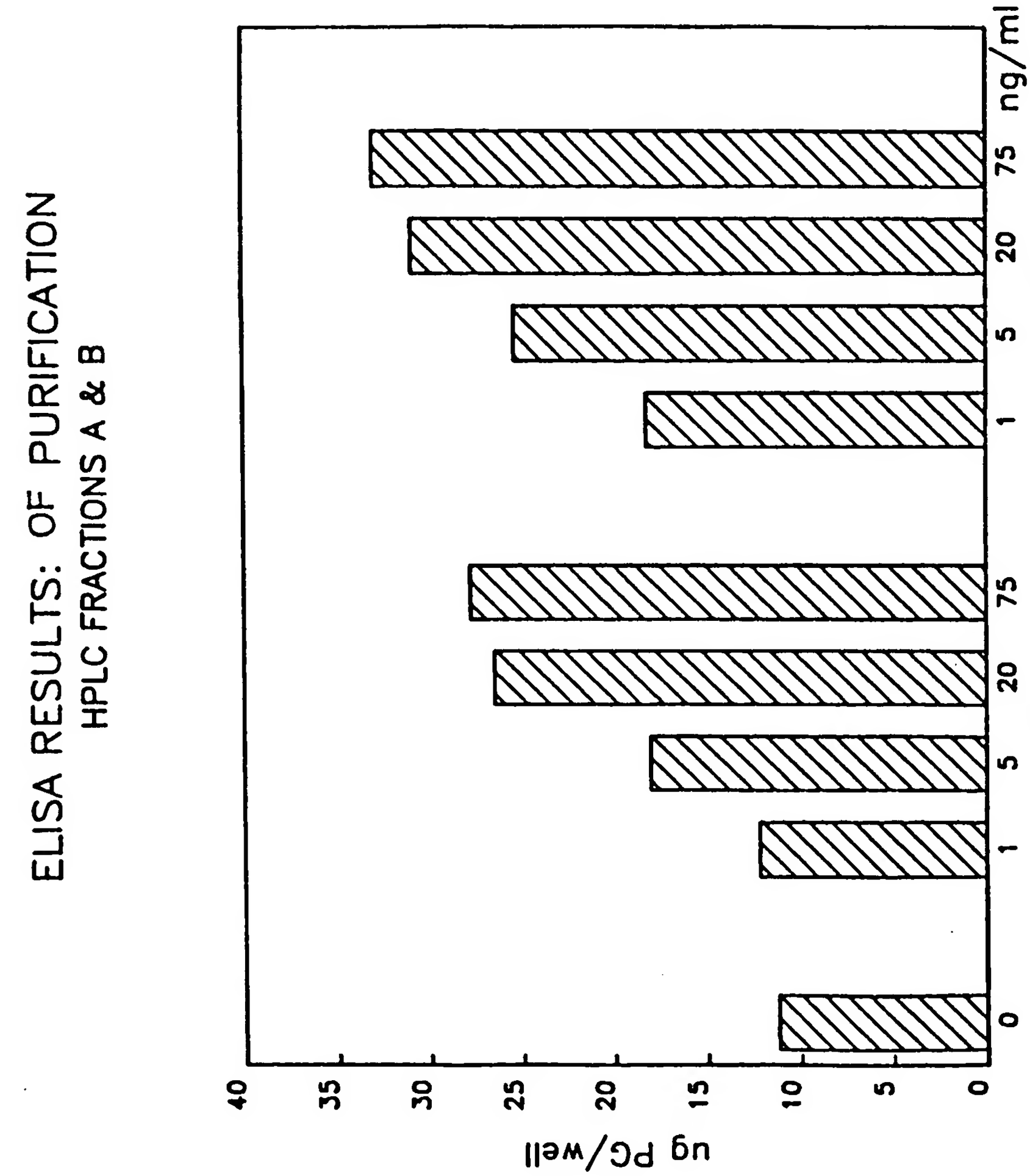
ELISA RESULTS: OF PURIFICATION
HPLC FRACTIONS A & B
ug PG/well
40
35
30
25
20
15
10
5
0
0
1
5
20
75
A
1
5
20
75
B
ng/ml

FIG. 4

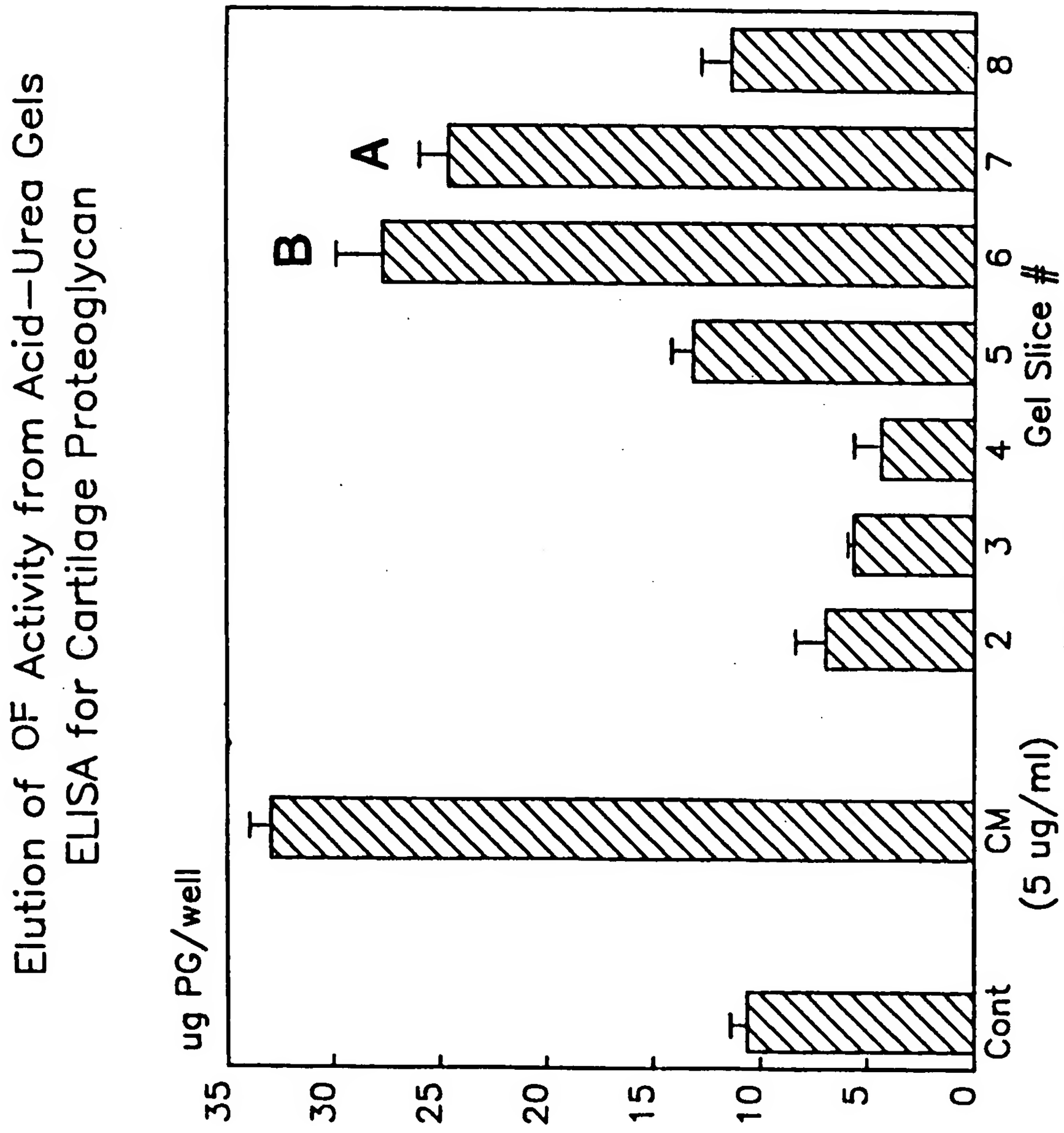
Elution of OF Activity from Acid–Urea Gels
ELISA for Cartilage Proteoglycan
ug PG/well
35
30
25
20
15
10
5
0
Cont
(5 ug/ml)
CM
2
3
4
5
Gel Slice #
6
7
8
A
B

FIG. 5

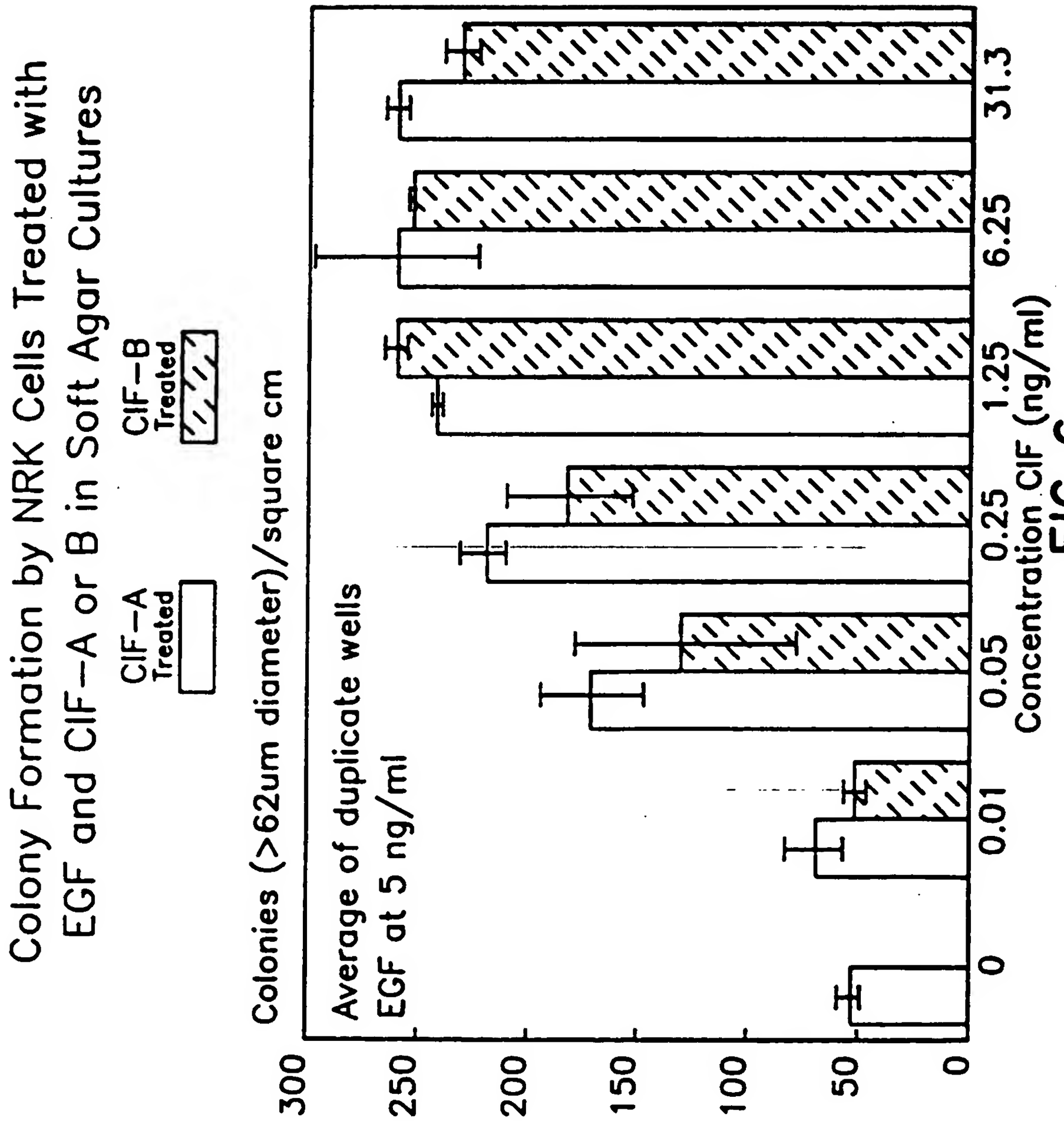
Colony Formation by NRK Cells Treated with EGF and CIF–A or B in Soft Agar Cultures
CIF–A Treated
CIF–B Treated
Colonies (>62um diameter)/square cm
Average of duplicate wells
EGF at 5 ng/ml
300
250
200
150
100
50
0
0.01
0.05
0.25
1.25
6.25
31.3
Concentration CIF (ng/ml)

FIG. 6